LINKING PROTEOMICS TO MICROBIAL KINETICS

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Abstract

Oligobacterial physiology is mostly unstudied due to cultivation difficulty. New isolation techniques such as extinction culture have produced cultivable representatives of the aquatic environment namely *Sphingopyxis alaskensis*. Attempts were made to grow the bacterium in batch cultures using glucose and tyrosine as ideal substrates as determined from growth studies. Differential protein expression from cytoplasmic and membrane fractions of the putative culture were compared so as to identify key proteins involved in substrate uptake and metabolism followed by incorporation of protein quantities into mathematical models of oligotroph growth. However artifactual results from two dimensional gel electrophoresis led to the question of culture purity, which was eventually confirmed by light microscopy, flow cytometry and 16S rRNA gene sequencing. This research gives better insight into the possible problems that can crop up while working with hard to culture marine oligobacteria. I demonstrate the rationale used to identify the contaminant, which was difficult to detect because its slow growth was similar to the target organism. A major achievement was successful cell fractionation as it has never been attempted in oligobacteria due to culturing difficulties and the procedure is different from the routine methods adopted in bacteria and fungi. Also the research demonstrates a complete protocol for eliminating uncertainties in culture purity.

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Chapter One

1. Introduction and Literature Review

Oligobacteria, which are bacteria adapted to low levels of nutrient concentration, play a major part in biogeochemical cycles in aquatic systems. Their major food source is dissolved organic matter and they produce carbon dioxide, minerals and cellular biochemicals.

The oceans are vast oligotrophic (Weber, 1907) environments that have the highest cellular production rate of any ecosystem on the planet. Despite the low level of nutrients in oligotrophic ocean waters, microbial numbers persist in the range 10^4 - 10^6 cells per ml. The fixation of carbon, nitrogen and phosphorus by bacteria and their conversion into particulate matter forms the basis of the microbial food web in the oceans. While these are critically important processes in aquatic environments, they are poorly characterized.

Downward flow of particles is the most efficient means of transporting carbon dioxide fixed by primary production to marine sediments, thus sequestering it from the atmosphere. The balance between particle degradation, regenerating carbon dioxide via respiration, and burial, is a critical factor affecting climate change and increases in ocean oligotrophy have been predicated to be a consequence of global warming (Jeffree & Szymczak, 2000). It is therefore important to have a better understanding of the genomic and proteomic makeup of the marine oligotroph organisms, as well as their ecophysiology.

A major characteristic of marine oligotrophs is their ability to adjust their metabolic system content despite possessing a small genome and cell size when compared to copeotrophs (Button, 2004). One of the major problems of understanding this mostly unseen but major component of the environment is its difficulty in isolation. Novel isolation techniques such as extinction culture (Button et al., 1993) and high throughput culturing (Connon & Giovannoni, 2002) have made it possible to culture these organisms under laboratory conditions. Partial genomic sequence and some proteomic studies (Fegatella et al., 1999) of *Sphingopyxis alaskensis*, a major oligotroph has been completed.

1.1 Microbial Kinetics

Microbial kinetics specify the growth rate of microbial cells as a function of dissolved substrate concentrations and other cell properties. The affinity of cells for substrate is usually expressed as the Michaelis constant K_m, which is the half saturation constant derived for isolated enzymes in transient phase systems (Michaelis and Menten, 1913). However this constant does not account for changes in the number or type of transporter proteins like permeases and porins in cells. Following hyperbolic kinetics used for single enzymes is not appropriate for whole cells as evident from unreasonably small K_m values of 10^{-10} M (Button, 1985) derived from substrate uptake studies. Uptake rates that fill metabolic pools or deplete external substrate may not be the same as bulk substrate concentrations, except for lower values of the latter.

Blackman's proposal (Koch, 1998) suggests that nutrient flux can be sharply limited by the concentration of down-stream enzymes This brings into question the Michaelis

Menten equation for substrate uptake as expressed by Monod (Monod, 1942), evidence for which is available in the case of light limited chlorella and some heterotrophic systems (Dabes et al., 1973).

The current state of microbial kinetics is presented here. A relationship between the rate of substrate uptake at a particular concentration and specific affinity as the intervening rate constant was modeled from the rate of collision between two particles:

$$v_p = k_p S$$

where, k_p is the particle based constant and S is the concentration of substrate

Specific affinity theory (Button, 1998), which relates the rate of transport per unit volume of cell material to substrate concentration and biomass, was derived from the above collision frequency theory.

The observed rate, $v_v = a_s S X$

where a_s, is specific affinity, and S and X represent substrate and biomass concentrations, respectively.

Value of the specific affinity can be determined by the following equation:

$$a_s = R_m (N_a 4 D r_s M / (4/3)\pi r^3_x \rho)$$

where R_m, the resistance of outer membrane to molecular diffusion of substrate as ameliorated by porins, D the molecular diffusion constant for substrate S, M the molecular weight , r_x radius of the cell, r_s radius of permease and ρ the organism density.

N_a, the number of active transporters among N total permeases, can be calculated as follows:

$$N_a = N \Delta \Psi^n / (K^n_{HS} + \Delta \Psi^n)$$

where $\Delta \Psi^n$, is the change in membrane potential, K^n_{HS}, the proton substrate interaction constant and n the number of interacting species (Button, 1998, 2004). Modeling of metabolic systems with respect to the structure, control and optimality (Heinrich and Schuster, 1998) and the concept of Combined Response Coefficient (de Vienne et al., 2001) also contribute to understanding of cellular kinetics.

1.2 Proteomics

A proteome is defined as the complete profile of proteins expressed in a given tissue, cell or biological system at a given time. Proteomics is the systematic analysis of the protein expression of such proteomes (Anderson and Anderson, 1997). Present proteomics evolved from protein chemistry with the advent of genomics data and protein identification tools such as mass spectrometry (Yates, 1999). Even though there are various approaches to explore a proteome, the most accepted approach has been one involving two dimensional gel electrophoresis (O`Farrel, 1975). High resolution two dimensional gel electrophoresis (O`Farrel, 1975) can reveal almost all proteins in a cell at any given time, including post translational modifications.

1.2.1 2D Gel Electrophoresis- Concept

A brief history and the chemistry involved are discussed below to have a better understanding of the technique.

1.2.1.1 History

In the mid 1970`s Patrick O`Farrel developed the first 2D Gel Electrophoresis system, where the isoelectric focusing for first dimensional separation was done using thin polyacrylamide tube gels. The gels comprised of urea, detergents, reductants and carrier ampholytes to form the pH gradient in the electric field. The sample was loaded onto the cathodal side of the gel rod, which is the basic end of the gradient in the electric field. The tubes were then equilibrated in an equilibrium buffer consisting of sodium dodecyl sulfate to impart negative charge to the separated proteins before loading it for the second dimension separation based on molecular weight. This technique was used for about two decades without much modification. Anderson & Anderson (1997) later designed a system to run multiple 2D gels under most reproductive conditions to develop a Human Protein Index. For proteome projects, gels with 20x20-cm size are standard for adequate spatial resolution. If one assumes that up to 100 bands can be resolved in a 20-cm 1D gel, a theoretical representation of 10,000 proteins could be obtained. In practice several thousands alone can be detected.

In O`Farrel tube gels, pH gradients are created by carrier ampholytes, a mixture of a few hundred different homologues of amphoetric buffers synthesized in one reaction flask. The mixture contains buffers with iso-electric points evenly distributed over a wide spectrum from pH 3 to 10. Their specialty is a high buffering power at their iso-electric point. At the beginning all carrier ampholytes are charged. When the electric field is applied they start to migrate according to their charge towards the anode or cathode respectively and form stable pH gradients between the electrodes. When they

reach their pI they lose their charge and buffer the pH at their pI. This technique works very well for native separations. But since long denaturing conditions are applied for high-resolution 2D techniques, long migration times are needed, which lead to destabilization of the gradient.

This first dimension step with carrier ampholytes was cumbersome as the gradient change with time reproducibility is affected. Batch to batch variations of the carrier ampholyte mixture was another concern. Loss of almost all basic proteins and some acidic proteins was observed as a result.

O`Farrel found a remedy for this loss of basic protein by making a modification of the first dimension separation by NEPHGE (Non Equilibrium pH Gradient Electrophoresis) (O`Farrel, 1977). Here the sample is loaded at the acidic end of the gel and proteins are separated as the gradient drifts toward the cathode. However due to time factor, good reproducibility was hard to achieve. Gel resolution depends on the number of different carrier ampholyte homologues. Proteins are stacked between the different carrier ampholyte homologues in NEPHGE.

To overcome these problems Bjellqvist et al., came up with an alternative to carrier ampholytes called IPG (Immobilized pH Gradient) (Bjellqvist et al., 1982) (Molloy, 2000). Here the pH gradients are prepared by co-polymerizing acrylamide monomer with acrylamide derivatives containing carboxylic and tertiary amino groups. Since the buffering groups forming the pH gradient are fixed, the gradient cannot drift and is not influenced by sample composition. In practice, thin slabs are co-polymerized with a film support, whose surface has been treated to covalently bind with the gels.

Later these film supported gel slabs are cut into individual strips for first dimension Iso-electric focusing.

1.2.1.2 Theory of Isoelectric Focusing

IEF is performed in a pH gradient. Proteins are amphoetric molecules with acidic and basic buffering groups. They become protonated or de-protonated depending on the pH environment. In basic environment, the acidic groups become negatively charged whereas in acidic environment the basic groups become positively charged. The net charge of a protein is the sum of all negative or positive charges of amino acid side chains and N and C terminus. The net charge can be plotted over the pH scale. The net negative charge curves are called titration curves. Each protein has an individual net charge curve. The intersection of the net charge curve with x-axis is the pI, which is the pH value where net charge is zero.

When a protein is placed at a certain pH value of the gradient and an electric field is applied, it will start to migrate towards the electrode of the opposite sign of its net charge. Because it migrates inside a gradient, it will arrive at a pH value of its isoelectric point. At the pI it has no net charge anymore and stops migrating. If it diffuses away above or below its pI it becomes charged again and migrates back to its pI. This is called a focusing effect, and it results in high resolution.

1.2.1.3 Second Dimension: SDS-PAGE (Sodium Dodecyl Sulfate- Polyacrylamide Gel Electrophoresis)

This technique separates the proteins according to their molecular weight (M_r). SDS and proteins form complexes with a necklace like structure composed of protein-decorated micelles connected by short flexible polypeptide segments. As a result of the necklace structure large amounts of SDS are incorporated in the SDS-protein complex in a ratio of approximately 1.4g SDS/g of protein. SDS masks the charge of proteins and thus formed anionic complexes have a roughly constant net negative charge per unit mass.

All protein will migrate towards anode (even though with basic pI). The electrophoretic mobility of protein treated with SDS and dithiothreitol (DTT) depends largely on the molecular weight of the protein. At a certain polyacrylamide percentage there is an approximate linear relation between log of M_r and relative migration distance of the SDS polypeptide complex of a molecular weight range. The molecular weights of sample proteins can be determined with the help of co-migrated standards with known molecular weights. SDS-PAGE does not give the absolute molecular mass of a protein but only gives an estimate. Exact mass can be found only by mass spectrometry (Yates et al., 1999).

Standard buffer systems for 2D SDS-PAGE are based on the discontinuous tris-chloride/ tris-glycine system described by Laemmli (Laemmli, 1970). The classical procedure according to O`Farrel involved a stacking gel with tris chloride pH 6.8 polymerized on top of the resolving gel, which contains tris-chloride pH 8.8. However

for 2DGE the stacking gel is not required since a one-dimensional separation by IEF has already been completed. Besides, gel-gel transfer of proteins is more efficient than liquid-gel transfer.

The high mobility chloride in the gel and the low mobility glycine in the running buffer help in stacking effect of the protein. The running buffer and gels contain 0.1% SDS. Sometimes the gels are cast without SDS, because the SDS migrating from the cathodal buffer into the gel is sufficient. During electrophoresis the negatively charged Cl^- , SDS and glycine migrate towards the anode, and positively charged Tris ions migrate towards the cathode.

1.2.1.4 Sample Preparation

This is the most sensitive step in the entire procedure. Identification of all proteins will be impossible if an error occurs in this stage. Modifications of proteins lead to wrong conclusions in proteome analysis. To avoid protein losses, the treatment of sample must be kept to a minimum. To avoid protein modifications, the sample should be kept on ice. To avoid losses and modifications, the preparation time should be kept as short as possible. A single protocol for all proteins is presently impossible, hence depending on the protein or protein group of interest alternative procedures have to be tried.

Once proteins have been extracted from the source material, they must be prepared for 2-D PAGE. The major goal of sample preparation is to solubilize as many proteins as possible to maintain their solubility throughout the 2-D PAGE process.

According to current practice, proteins are denatured to their constituent polypeptide chains so that the polypeptide sequences can be matched to their corresponding gene sequences. Secondary sample sample preparation concerns are the removal of non-proteinaceous material that may interfere with 2-D PAGE and the prevention of artifactual modifications of polypeptides.

Proteins are extracted from source material by well established cell disruption methods, then solubilized and denatured by means of chaotropes, detergents, and reducing agents.

Chaotropes such as urea and thiourea are employed at high concentrations to disrupt hydrogen-bonded structures in the sample proteins. Non-ionic or zwitterionic detergents are used to disrupt hydrophobic interactions. CHAPS, triton X-100 and newer detergents, such as sulfobetaine SB3-10 and amidosulfobetaine ASB-14 are popular IEF compatible additives. Reducing agents dithiothreitol (DTT), and tributyl phosphine (TBP) reduce disulfide bonds (cystine residues) to sulfhydryls (cysteine residues) .

A good solubilization solution for most purposes consists of 8-9 M urea, 4% CHAPS, 50mM DTT and 0.2% (w/v) carrier ampholytes and a trace of bromophenol tracking dye.

Sequential extraction is an effective way to compartmentalize proteins in terms of their solubility. It constitutes a third dimension of separation. In this procedure proteins are extracted into solutions of increasing solubilization power and then separated by

2-D PAGE. Insoluble proteins remaining from one extraction are treated with the next solution in the sequence.

Nucleic acids interfere with efficient IEF separations by both binding to proteins and increasing the viscosity of the sample solution, The usual way of dealing with nucleic acids is to degrade them with nucleases prior to IEF. Non specific micrococcal nuclease or a mixture of pancreatic RNase and DNase added to the extraction mixture usually degrades nucleic acids sufficiently (Garfin, 2003).

Polysaccharides in the sample solutions are not severely detrimental to IEF. They are usually ignored, mainly because there is no simple method for removing them. The best way to remove lipids from samples appears to be by precipitating the proteins with organic solvents (Mastro & Hall,1999).

1.2.1.5 Protein Concentration Determination

To get a reproducible gel, the protein concentration should be constant and optimum. Protein quantitation was done by several different assays. These included

(a) Bradford method (Bradford, 1976), which is the preferred method for quantifying proteins. This technique is faster, simpler and more sensitive than the Lowry method. Moreover, when compared with the Lowry method, it is subject to less interference by common reagents and non-protein components of biological samples. This assay relies on the binding of the dye Coomassie blue G250 to protein. The quantity of protein can

be estimated by determining the amount of dye in the blue ionic form by measuring absorbance of the solution at 595 nm.

The dye appears to bind most readily to arginyl and lysyl residues of proteins. This specificity can lead to variation in the response of the assay to different proteins, which is the main drawback of the method. Two types of assays are available under this method- the standard assay (suited for measuring between 10 and 100 μg protein) and the microassay (for detecting between 1 and 10 μg protein).

(b) Lowry Method (Lowry et al., 1951)- This method is based on both the Biuret reaction, where the peptide bonds of proteins react with copper under alkaline conditions producing Cu^{+}, which reacts with the Folin reagent, and the Folin-Ciocalteau reaction which is poorly understood but in essence phosphomolybdotungstate is reduced to heteropolymolybdenum blue by the copper catalyzed oxidation of aromatic amino acids. The reactions result in a strong blue color, which depends partly on the tyrosine and tryptophan content.

(c) BCA Method (Smith et al., 1985) -This was first described by Smith et al and is similar to Lowry assay, since it also depends on the conversion of Cu^{2+} to Cu^{+} under alkaline conditions. The Cu^{+} is then detected by reaction with BCA (bicinchoninic acid). This has the advantage of having one step less than the Lowry assay. Both standard assay (0.1-1.0 mg protein/ml) and microassay (0.5-10 μg protein /ml) are available.

1.2.1.6 Detection Methods and Image Analysis

Currently there are over ten methods to stain proteins of which silver staining, Colloidal Coomassie Blue (CCB), SYPRO Ruby and radioactive labeling are the most sensitive and preferred ones.

For proteomics work, protein stains must be compatible with Mass Spectrometry (MS) and that has limited the choice of silver stain to those that do not include glutaraldehyde treatment or oxidation steps. Coomassie Blue and SYPRO Ruby stains are both compatible with MS. CCB is an end point stain, meaning that gels can be left in it overnight for convenience. A post stain water wash is important with CCB. The wash removes excess colloidal dye particles from the gel surface and also drives the dye molecule into the proteins in the gel. Thus the wash increases the signal to noise ratio of the stained gel. CCB is the least sensitive of the stains mentioned above. Its detection limit is about 10 ng of protein per spot. CCB stains a wide range of proteins and responds linearly over two orders of magnitude in protein amount. Protein spots turn blue on staining with CCB and gel images are captured with a scanning densitometer (visible light).

SYPRO Ruby is a fluorescent stain. It is also an end point stain that can detect proteins at about 1 ng per spot. Depending on the proteins involved, it can be linear in response over three orders of magnitude in protein amount. A fluorescent imager like Typhoon is necessary for visualizing the spots.

Silver staining (Rabilloud, 1999) is the most sensitive non-radioactive method for protein visualization, enabling protein spots containing less than 1ng to be detected. The

range of linearity is less than two orders of magnitude. All silver staining methods are very temperature dependent. They require several precisely timed manipulations and are very subjective when it comes to deciding when to stop development. Hence it is the least reproducible of the stains.

There is presently no known universal stain that will react equivalently with all of the proteins in the gel. It is, therefore valuable to stain with more than one kind of procedure at some stage. Double staining is possible and has been done with CCB and silver.

Image acquisition instruments range from simple cameras and light boxes to sophisticated laser-based fluorescence detectors. For subsequent digital analysis, gel images should be captured electronically. The three categories of image acquisition devices used with 2-D PAGE are document scanners, charge coupled device (CCD) cameras and laser-based detectors. Document scanners (densitometers) operate in visible light and are used when gels are stained with CCB or silver. CCD cameras can be used either visible or fluorescent stains.

With 2-D PAGE gels of any size, image resolution of 100-150 μm is adequate. For best results the imager should be matched to the software used for analysis. Image analysis (Miller, 1989) (Yan et al., 1999) is the heart of proteomics research. It provides the analysis and control functions necessary to integrate and manage the various separation and analysis processes. Although gel images can be examined visually, objective quantification and comparison of the large number of protein spots require computer assistance. Software for 2-D PAGE analysis defines and quantifies spots in 2-

D gels, removes background patterns, matches images from related gels, compares the intensities of corresponding spots in related gels (quantitative changes in expression), prepares gel data for presentation and exports gel-image information to databases.
Image analysis software also guides the excision of proteins from gels for further analysis, whether this is done manually or automatically with a spot excision robot.

1.2.1.7 Protein Identification and Quantification

Excised spots are digested using a proteolytic enzyme trypsin and purified for mass spectrometry sample preparation (Shevchenko et al., 1996). Mass spectrometry enables protein structural information, such as peptide masses or amino acid sequences to be obtained. This information can be used to identify the protein by searching nucleotide and protein databases. The technique involves three stages (1) sample preparation, (2) sample ionization, and (3) mass analysis.

Matrix assisted laser desorption ionization (Karas et al., 1988) was to be the preferred technique followed by a peptide mass fingerprinting (James et al., 1993) database search utilizing the genome sequence information recently published.
Protein Quantification is possible by either use of traditional biochemical techniques like C-14 labeling or by use of recent developments enabled by advancements in Mass spectrometry such as ICAT (Isotope coded Affinity Tag) (Goodlett et al., 2001) and iTRAQ (Isobaric Tagging) (Thompson et al., 2003).

Protein-protein interaction studies using chemical crosslinkers, affinity capture strategies, microarrays, invivo flourescence resonance energy transfer (FRET) and yeast

two hybrid analysis have already opened up the gateway of systems biology to model organisms, however so far, not for the common and more important oligotrophs.

1.3. The Candidate- *Sphingopyxis alaskensis*

The marine oligobacterium *Sphingopyxis alaskensis* was isolated (Schut et al., 1993) in Resurrection Bay near Seward, Alaska and in the central North Sea off the Dutch coast and cultured in laboratory conditions using filtered autoclaved seawater following dilution to extinction. It was demonstrated that it is one of the most abundant culturable inhabitants from the oceans and is thought to be a major contributor to microbial biomass in marine waters and also its role in nutrient cycling. Two strains RB2256 (Schut et al., 1997) in particular and AF01 (Eguchi et al., 2001) to a lesser extent have been studied in detail to understand the basis of microbial oligotrophy. Studies have shown that their capacity to thrive in oligotrophic environments is linked to unique genetic and physiological properties which are fundamentally different from those of well studied copiotrophic prokaryotic models such as *Escherichia coli.*

Its unique physiology is attributed to a high affinity, broad specificity uptake, simultaneous multiple substrate uptake, high level of oxidative stress resistance (Fegatella and Cavicchioli, 2000), and the ability to convert substrates into biomass during slow nutrient limited growth. Cross-species identification of proteins (Ostrowski et al., 2004) from proteome profiles of this organism was attempted while the genome was being sequenced to better understand the proteins involved and thereby the physiology. Analytical reference maps were generated from mid log phase batches resolving up to 1600 spots. Even though the above attempts were successful in

indicating the type of proteins composed, a better understanding of substrate to biomass conversion can be better explained through a link between microbial kinetics and the cytoarchitecture of the organism.

1.4. Cytoarchitectural Model of Nutrient Kinetics

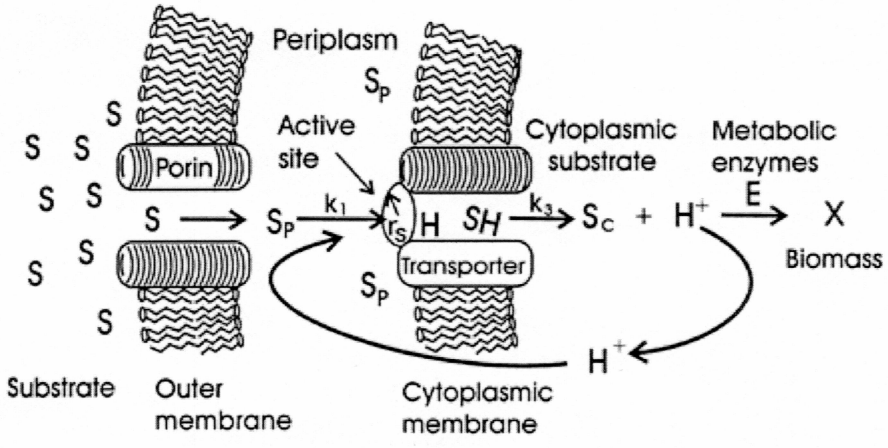

Fig 1. Cytoarchitectural model of nutrient kinetics (Button et al., 2004)

The above figure is a transthreptic cell where ambient nutrient S diffuses through a porin to periplasmic concentration where it combines with a proton-empowered permease having an active site radius of r_s. Transport produces a flux that generates both a proton potential and a concentration driving force that facilitates substrate flow through an enzyme sequence (E) to produce cell material (X).

The use of this model can be explained by the formulations relating rate to cytoplasmic and membrane proteins discussed earlier. In addition to qualitative

identification of the proteins by MALDI-TOF, quantitative estimation of the number of metabolic and membrane proteins can be made by use of radiolabeled substrates. A differential expression study using a carbohydrate substrate and amino acid substrate can help to understand the change in cytoarchitecture with substrate and thereby the rate.

Some of the other questions that could be answered are as follows: (a) *Starvation proteins*. Chaperones and other proteins often appear during starvation in part to preserve the tertiary structure of enzymes. Are these proteins, known to be present in oligobacteria a significant cost to survival at small concentrations of substrate that sustain at low rates of growth? (b) *Permease enzyme ratio*. How are available resources parsed between permeases and cytoplasmic enzymes to obtain observed values for specific affinity? (c) *Metabolic enzymes*. What is a typical number for important cytoplasmic enzyme molecules per cell needed at usual concentrations in the metabolic pools for various rates of growth? (d) *Minimum cell size*. What is the minimum size of a cell that can exist under oligotrophic conditions? (e) *Viability*. Reasons for low apparent viabilities of aquatic bacteria and the sometimes inhibitory influence of added substrates remain unclear. Clues to this dilemma could perhaps be found in the proteomes of marine bacteria when perturbed with chemical amendments.

Chapter Two

2. Materials and Methods (See Appendix F for source of chemicals)

2.1 Cell Cultures

2.1.1 Growing Putative RB2256 as a Maintenance Culture

A maintenance batch liquid culture was made every fortnight as follows. Media was made by pouring 50 mL of Artificial Sea Water (ASW) (See Appendix A) pH 7.8 into an Ehrlemeyer flask followed by addition of 20 mL Nutrient Broth and then plugged with cotton. Final pH adjusted around 7.8, autoclaved at 121 ° C for 40 minutes and cooled to room temperature. This was followed by addition of sterile KH_2PO_4 (140 µL) (See Appendix C), trace mineral mix (70 µL) (See Appendix D), and vitamin premix (70 µL) (See Appendix B) flaming each time the mouth of the flask. The media was then inoculated with 100 µL of RB2256 and kept on a shaker.

2.1.2 Growing Putative RB2256 in a Carbohydrate Source, Glucose.

A glucose based liquid culture was made as follows. Media was made by pouring 100 mL of Artificial Sea Water (ASW) pH 7.8 into an Ehrlemeyer flask. 0.01 g of D glucose was added to the above. Final pH was then adjusted around 7.8, the flask plugged with cotton, autoclaved at 121 ° C for 40 minutes and cooled to room temperature. This was followed by addition of sterile KH_2PO_4 (200 µL), trace mineral mix (100 µL) and vitamin premix (100 µL) flaming each time the mouth of the flask. The media was then inoculated with 100 µL of RB2256 from a maintenance culture and kept on a shaker.

2.1.3 Determining an Amino Acid Source for Growing Putative RB2256

Twenty test tubes were taken and labeled according to the amino acid to be added. 20 mL of ASW was added into each tube and the amino acids in their respective labeled test tubes @ 0.1 g per liter. The amino acids used were as follows, glycine, alanine, valine, phenylalanine, methionine, leucine, isoleucine, proline, glutamine, lysine, tyrosine, arginine, serine, histidine, tryptophan, cysteine, asparagine, threonine, glutamic acid and aspartic acid. Turbidity of cultures were compared to determine the best candidate as an amino acid substrate.

2.1.4 Putative Growing of RB2256 in an Amino Acid Source, Tyrosine.

A tyrosine based liquid culture was made as follows. Media was made by pouring 100 mL of Artificial Sea Water (ASW) pH 7.8 into an Ehrlemeyer flask. 0.01 g of L tyrosine was added to the above. Final pH was then adjusted around 7.8, the flask plugged with cotton, autoclaved at 121 ° C for 40 minutes and cooled to room temperature. This was followed by addition of sterile KH_2PO_4 (200 µL), trace mineral mix (100 µL) and vitamin premix (100 µL) flaming each time the mouth of the flask. The media was then inoculated with 100 µL of RB2256 from a maintenance culture and kept on a shaker. Larger volumes of glucose and tyrosine cultures were grown by scaling up to one liter.

2.2 Purity of Cultures

2.2.1 Agar Plates

Purity of cultures were confirmed by use of agar plates. To about 500 mL of ASW (pH 7.78) 12.5 g bacteriological agar, 0.05 g casaminoacids were added. 0.05 g D glucose and tyrosine were added to the glucose and tyrosine based media respectively. The above ingredients were mixed and autoclaved at 121 ° C for 20 minutes.

The media was then poured directly into the plates up to half the volume. After cooling the plates were inoculated by making streaks from the respective liquid cultures. Plate cultures were observed for possible foreign colonies.

2.2.2 Epifluorescence Microscopy

Epifluorescence microscopy was used as a tool to confirm absence of contamination.

Stock solutions and setup

Stock solutions of 5 % triton X-100 stored in dark in 1mL aliquots and DAPI (0.5mg/mL) stored in dark in 0.5 mL aliquots were made. Fresh staining solution was prepared by first thawing the stock solutions and adding 50 µL DAPI to the 1 mL aliquot of triton X-100 and filtered. 20 µL of the filtered staining solution was added per 1mL of the diluted sample (ratio of sample to water being1:1000). The mixture was shaken well in a vial and incubated in the dark for an hour. Vacuum was set up and black 0.1 µm pore size polycarbonate filters with 25 mm diameter was applied to the frit with the shiny side up. Filter extension was clamped and sample added to filter before turning on the

vacuum. The vacuum was turned on but care taken not to dry the filter. 2-3 drops of filtered distilled water were used to rinse the filter.

A slide was prepared by first spreading immersion oil evenly over the slide followed by placing the filter with the shiny side up and flat onto the oil layer. A drop of oil was placed onto the filter followed by a cover-slip on top. Finally a drop of oil was placed onto the cover-slip, the slide labeled and ready for observation under the microscope using a 100X lens. Cells were collected from glucose and tyrosine based liquid cultures and plates as well.

2.2.3 Flow Cytometry

Samples for DNA content analysis were prepared using 1 mL of each of the culture to which 12 μL of filtered formaldehyde was added (Button and Robertson, 2001). To account for differences in the G+C contents of species due to the AT binding specificity of DAPI, the apparent DNA content was adjusted with reference to E. coli (51.75 G + C mol%) and assumed a G+C content for *Sphingopyxis alaskensis* (RB2256) of 65 mol % (Button & Robertson, 2001).

2.2.4 16S r DNA Sequencing

2.2.4.1 DNA Extraction

Initial attempt was made to isolate whole cell DNA by taking 9 mL of the cultures into Beckman ultra clear centrifuge tubes and spinning in a Beckman L8 70M ultra-centrifuge at 36,000 rpm using an SW40 rotor at 4 ° C for 60 minutes. The supernatant

was discarded and the pellets retained. The bacterial cells were lysed by freeze-thaw cycles (-20 ° C for 5 minutes, room temperature for 5 minutes, 5 cycles).

2.2.4.2 Polymerase Chain Reaction (PCR) Set Up

Partial amplification of 16S rDNA was done as follows. See Appendix E for primer sequence.

1. *Set up the PCR reactions:*

Upstream primer EUBf933	2 µL
Downstream primer EUBr1387	2 µL
$MgCl_2$ (25 mM)	3 µL
PCR nucleotide mix (10 mM each)	1.5 µL
Thermophilic DNA polymerase 10x reaction buffer	5 µL
Taq DNA polymerase (5 µg/µL)	1 µL
Template DNA	10 µL
Nuclease-free water to a final volume of	25.5 µL

2. *Set up the program for PCR:*

	Temperature (°C)	**Time (minutes)**	**Cycles**
Initial denaturation	95	2	1
Denaturation	95	1	30
Annealing	50	1	30
Extension	72	1.5	30
Final extension	72	5	1
Soak	4	indefinite	

2.2.4.3 PCR Product Detection

1. 1500 mg agarose was added into 100 mL 1x electrophoresis TBE (tris/ borate /EDTA) buffer, mixed and heated in a microwave for 2 minutes.
2. 100 µL of 1 g/L ethidium bromide was added to the gel. The concentration of ethidium bromide was 1 µL/ mL.
3. This agarose gel was cooled until it is about 60° C.
4. 1.5% agarose was casted in the tray until it solidified.
5. 10 µL PCR products were loaded with 5 µL loading dye on the 1.5% agarose gel.
6. The gel was run at 90 volts for 1 hour.
7. A UV scanner was used to capture the gel image.

PCR products not being found on the gel, a QIAGEN DNA easy extraction kit was used. Also a nanospectrophotometer was used to quantify the DNA isolated.

2.2.4.4 PCR Product Purification

1. The Microcon-PCR sample reservoir was inserted into one of the two vials provided.
2. 460 µL autoclaved distilled water or TE buffer was pipetted into sample reservoir without touching the membrane with the pipette tip.
3. 40 µL PCR product (aqueous phase only) was added to the reservoir and sealed with attached cap.
4. The Microcon-PCR unit was spun at 1000 g (3500 rpm) for 15 minutes.
5. Vial was separated from sample reservoir.

6. Sample reservoir was placed upright into a clean vial and added 20 µL autoclaved distilled water or TE buffer carefully to the reservoir.
7. The reservoir was inverted into a clean vial.
8. At 1000 g (3500 rpm) the vial was spun for 2 minutes to collect the DNA filtrate.

2.2.4.5 Cycle Sequencing

1. Set up cycle sequencing reaction:

    4.0 µL BigDye

    2.0 µL primer (10 pmol)

    3.0 µL 5x sequencing buffer

    2.0 µL DNA

    9.0 µL dd water

2. Set up cycle sequencing program:

    96 °C -1 minute

    96 °C -10 seconds

    50 °C - 5 seconds

    60 °C - 4 minutes

    x 25 cycles

2.2.4.6 Sephadex G-50 for Purification

1. Added 650 µL sephadex to the column.
2. Spun at 2.5 rpm for 3 minutes.
3. Placed column into 1.5mL centrifuge tube.
4. Pipetted 20 µL sample into sephadex matrix.
5. Spun at 2.5 rpm for 3 minutes.
6. Removed column, dry sample in Speed Vacuum (med heat, one speed).
7. Stored in freezer pending sequencing.

2.2.4.7 Sequencing with ABI 3100 Genetic Analyzer

1. 15 µL formamide was added and vortexed.
2. 96-well plate was used for transfer.
3. Thermal cycler was used to heat at 95°C for 3 minutes.
4. The 96-well plate was placed on ice immediately following heating.
5. ABI 3100 sequencer was used for sequencing and analysis of sequence was done using Sequencher v4.5.
6. BLAST was used to identify the organism based on the 441bp 16Sr RNA gene sequence.

2.3 Cell Harvesting

The culture was poured into centrifuge tubes in equal volumes and centrifuged @ 8310 rpm (10,000 * g) for 10 minutes at 4 ^{o} C in a Sorvall centrifuge. The supernatant

was removed with a Pasteur pipette and cell pellets were re-suspended in about 1 to 2 mL of ASW-EDTA buffer mix. The re-suspended cells were mixed into two lots and centrifuged again @8310 rpm for 10 minutes at 4 ° C in a Sorvall and the supernatant was removed. Cell pellets were re-suspended in a breaking buffer (final concentration of 50 mM sodium phosphate, 10 mM Hepes buffer (pH 7.8) and glycerol (20 % v/v). DNase and RNase @ 10 µL per mL each were added. Protease inhibitor was also added@ 150 µL per 100 mg of pellet. Finally 2 mL of breaking buffer was added to re-suspend the pellet in each tube and kept in ice for 2 hours.

2.4 Breaking the Cells and Membrane Separation

A French press was used where the cylinder was pre-cooled and the cell volume passed through a pressure of 16000 psi for 5 minutes. The broken cell fraction was centrifuged @ 4000 X g for 5 minutes. The supernatant was removed and stored in ice. $MgCl_2$ was added to a final concentration of 2 mM to the supernatant. The supernatant was centrifuged at 15000 X g for 30 minutes to obtain membrane fraction as pellet and cytoplasmic fraction as supernatant. The above steps were repeated on the remaining cell pellet.

2.5 Inner and Outer Membrane Separation

A sucrose gradient was made using 2.6 M sucrose stock solution at the following gradient levels- 90%, 85 %, 80%, 75 %, 70 % and 65 %. The volumes of the gradient

were 1.5 mL, 2mL, 2mL, 2mL and 1.5 mL respectively. Just above the 65% layer 2 mL of membrane fraction was added capped and ultra-centrifuged @ 36000 rpm for 25 hours (230,000 X g) using a Beckman SW-40 rotor.

2.6 Sample Preparation for 2D Gel Electrophoresis

The frozen fractions were thawed in ice and spun in Vivaspin 500 concentrators for 10 minutes @ 13,000 X g. The concentrate was then transferred to 1.5mL Eppendorf tubes and kept in ice. Protein quantification was followed using Lowry assay.

2D clean up kit from Biorad was used to remove the remnant impurities. 100 µL of the sample was transferred into a 1.5 mL microcentrifuge tube. 300 µL precipitating agent 1 was added to the protein sample and mixed well by vortexing followed by incubation on ice for 15 minutes. 300 µL precipitating agent 2 was added to the mixture of protein and precipitating agent 1 and mixed well by vortexing. The tubes were centrifuged at 12000 X g for 5 minutes to form a tight pellet. The supernatant was carefully removed without disturbing the pellet using a pipette. The tube was positioned in the centrifuge as before (cap hinge and protein pellet facing outward) and centrifuged for 15-30 seconds to collect any residual liquid at the bottom of the tube. 40 µL of wash reagent 1 was added on top of the pellet and positioned the tube in the centrifuge as before followed by centrifuging at 12,000 X g for 5 minutes. After removing the wash 25 µL of proteomic grade water was added on top of the pellet and vortexed for 10-20 seconds. 1 mL of wash reagent 2 (pre-chilled at -20 ° C for at least an hour) was added along with 5 µL of wash 2 additive. The tube was vortexed for one minute and incubated the tube at -20 ° C for 30 minutes. The tube was vortexed for 30 seconds every 10

minutes during the incubation period. After the incubation period, the tube was centrifuged at 12000 X g for 5 minutes to form a tight pellet. The supernatant was discarded followed by centrifugation for 15 seconds and discarding of remaining wash. The pellet remaining was air dried for less than 5 minutes and re-suspended in appropriate volume of 2D rehydration buffer.

2.6.1 First Dimensional Separation – Isoelectric Focusing

Procedure:

Passive rehydration: The IPG strips are rehydrated passively by placing them in a rehydration tray with the gel side down in 320 µL of the solubilized protein sample. Care was taken not to introduce any bubbles which would interfere with the even distribution of the sample in the strip. After half an hour or so the strips were overlaid with 2 mL of mineral oil to prevent evaporation during the rehydration process. The rehydration tray was covered with the plastic lid and the tray was left sitting on a bench level overnight. The next day using forceps the strips were gently washed with nanopure water and dried on a Kimwipe with the plastic side down to remove excess mineral oil. The strips were then placed in the lanes in a focusing tray with the plastic side up and oriented in such a way that the "+" mark on the strip is positioned at the end of the tray marked "+". The strips were then overlaid with 2 mL of mineral oil and the focusing tray was placed into the PROTEAN IEF cell and covered with the lid. The strips were pre-focused at 250 V for 15 minutes, focused at 10,000 V for 12-14 hours.

Focusing of the strips is followed by equilibration where they were first immersed in Buffer 1 prepared by mixing 6 M urea, 2 % SDS, 0.375 M tris-HCl pH 8.8, 20 % glycerol and 130 mM DTT for 10-15 minutes followed by immersing the strip in Buffer 2 prepared by mixing 6 M urea, 2 % SDS, 0.375 M tris-HCl pH 8.8, 20 % glycerol and 135 mM iodoacetamide for 10-15 minutes.

2.6.2 Second Dimensional Separation by SDS PAGE

2.6.2.1 Assembling the Gel Plates

Gel plates of dimension 20X20 cm were used. The gel sandwich was assembled on a clean surface. The long rectangular plate was laid first followed by placing the two spacers of equal thickness along the long edges of the rectangular plate. Next a short plate was placed on top of the spacers so that it remained flushed with one end of the long plate.

Both the right and left sandwich clamps were located and the single screw of each was loosened by turning counterclockwise. Each clamp was placed by the appropriate side of the glass next to the plate stack with the locating arrows facing up and towards the glass plates. The glass plate sandwich was grasped firmly with right hand and the left clamp was guided onto the sandwich with left hand so that the long and short plates fit the appropriate notches in the clamp. The single screw was tightened enough to hold plates in place. The right clamp was placed on the right side of the plates and the clamp screw tightened. The spacers being flushed well against the sides of the clamp was checked for. The casting stand was leveled on a flat surface with the alignment slot. The

assembled gel sandwich was placed in the alignment slot of the casting stand. The clamp screws were loosened and the plates and spacers were allowed to align at the surface of the alignment slot. While pushing down on one spacer with one finger, the clamp screw was tightened finger tight with the other hand. The aligned sandwich was placed into one of the casting slots with the longer plate facing me. When the sandwich was placed correctly the cams were pushed in and turned 180 degrees.

2.6.2.2 Casting the Gels

Acrylamide stock solutions (30% T, 2.6 % C) were made by dissolving 75 g acrylamide and 2 g of Bis acrylamide in 18M Ohm water up to 250 mL. Activated charcoal was added to this and filtered the next day using 0.45 μm filter paper. A homogeneous gel solution was prepared by mixing 33.5 mL of distilled water, 25 mL 1.5 M tris-HCl pH 8.8, 1mL 10 % (w/v) SDS stock , 40 mL acrylamide stock , 500 μL 10% fresh ammonium persulfate and 50 μL TEMED. The last two ingredients were added just before casting. 24 mL of the gel solution was poured into the space between the sandwich followed by adding a few drops of 1:1 isobutanol-water mix using a Pasteur pipette to make the surface of the gel even.

2.6.2.3 Running the Gels

The equilibration of strips is done usually only after the gel is polymerized. The polymerized gel was first washed with 18M Ohm water to remove the isobutanol-water traces. The strips were then placed vertically on the even gel surface without bubbles. An

agarose sealing solution (0.5% agarose M or NA, 0.01 % bromophenol blue and SDS cathode buffer up to a final volume of 100 mL) was used on top of the strip inserted. The Bio-Rad PROTEAN II slab cell is a vertical slab electrophoresis instrument which has a central cooling core to which the two gel sandwiches are attached. The outer plate of the sandwich forms the side of an upper buffer chamber. The inner plate was clamped against a rubber gasket on the central cooling core to provide a leak free seal for the upper buffer. The upper buffer chamber holds approximately 350 mL of buffer.

The lower buffer chamber of the PROTEAN II encloses the unit, provides stability during electrophoresis and requires 1.1 liters of buffer volume. The cooling core was filled with a mixture of methanol and water. A 5 X running buffer was made by adding 45g tris base, 216 g glycine and 15g SDS to 3 liter of 18 M Ohm water. Just before use, 300 mL of the 5X stock buffer was diluted with 1200 mL 18 M Ohm water for one electrophoresis run.

The lower chamber was first filled with approximately 1.2 liters of this diluted running buffer slowly followed by 350 mL of the remaining running buffer into the upper buffer chamber. The unit was closed with the lid and the electrodes connected to the Bio-Rad power source. Running conditions for the cytoplasmic and membrane fractions were as follows: 36mA for first half hour, 48mA for 7 hours 15 minutes for both the sandwiches together, 1404 Volt-hours.

2.6.2.4 Staining

Silver staining protocol was used to stain the protein spots. The gels after second dimensional run were carefully taken out and first immersed in a fixing solution (10 % acetic acid, 40 % ethanol with de-ionized water up to final volume of 500 mL). The gels were fixed twice in 250 mL each of the fixing solution for an hour.

They were then transferred to a sensitizing solution (75 mL ethanol, 10 mL sodium thiosulfate 5% (w/v), 17 g sodium acetate, de-ionized water up to final volume of 250 mL) for 30 minutes. This was followed by three 10-minute washing steps with de-ionized water after which it was transferred to a silver solution (25 mL silver nitrate (2.5%) in de-ionized water up to final volume of 250 mL). Two to three washing steps with de-ionized water follows 1 minute each. The gel was transferred to a developing solution (6.25 g sodium carbonate, 100 μL formaldehyde, and de-ionized water up to a final volume of 250 mL) for 4 minutes. As observing differential expression of proteins is the objective care was taken to run and stain the cytoplasmic proteins from tyrosine and glucose simultaneously. The same was followed to compare the pattern for membrane proteins. Staining of gels was stopped using 15 % acetic acid. The stained gels were scanned using an 8M pixel gel scanner.

Chapter Three

3. Results and Discussion

3. 1 Results

This chapter illustrates first the comparative growth of putative *Sphingopyxis alasknesis* in amino acids, followed by comparison of the cytoplasmic proteins of *Sphingopyxis alaskensis* grown under two different substrates using 2D Gel Electrophoresis. Also included is a gel representing successful separation of membrane proteins using a thiourea based solubilization approach. Simultaneously with the proteome studies the purity of culture is examined using epiflourescence microscopy, flow cytometry and 16S rDNA sequencing to avoid artifactual results.

3.1.1 Comparative Growth in Amino acids

Table 1. Comparative growth of putative *Sphingopyxis alaskensis* in amino acids. Table 1 shows major differences in the ability to grow on mineral media amended with each of 20 amino acids. Surprisingly, only tyrosine supported heavy growth as evident from the turbidity of the culture. '+' and '-' denote presence and absence of growth respectively.

Amino acid	Growth (+/-)	Amino acid	Growth (+/-)
Glycine	-	Lysine	-
Alanine	++	Histidine	-
Valine	-	Cysteine	-
Leucine	-	Threonine	-
Isoleucine	-	Tyrosine	+++
Methionine	-	Serine	-
Phenylalanine	-	Arginine	-
Glutamine	-	Proline	+
Glutamic acid	+	Tryptophan	-
Aspartic acid	-		
Asparagine	-		

3.1.2 Epiflourescence Microscopy

Small blue DAPI stained cells of size 0.8 μm were seen along with occasional bright yellow colored cell like bodies in a ratio of about 1: 10.

3.1.3 2D Gel Electrophoresis

Comparison of Fig 2 and Fig 3 shows differential expression of proteins under substrate induction. Though a similar pattern in protein spots is seen in the electrophoretograms an unusual number of protein spots seem to be expressed.

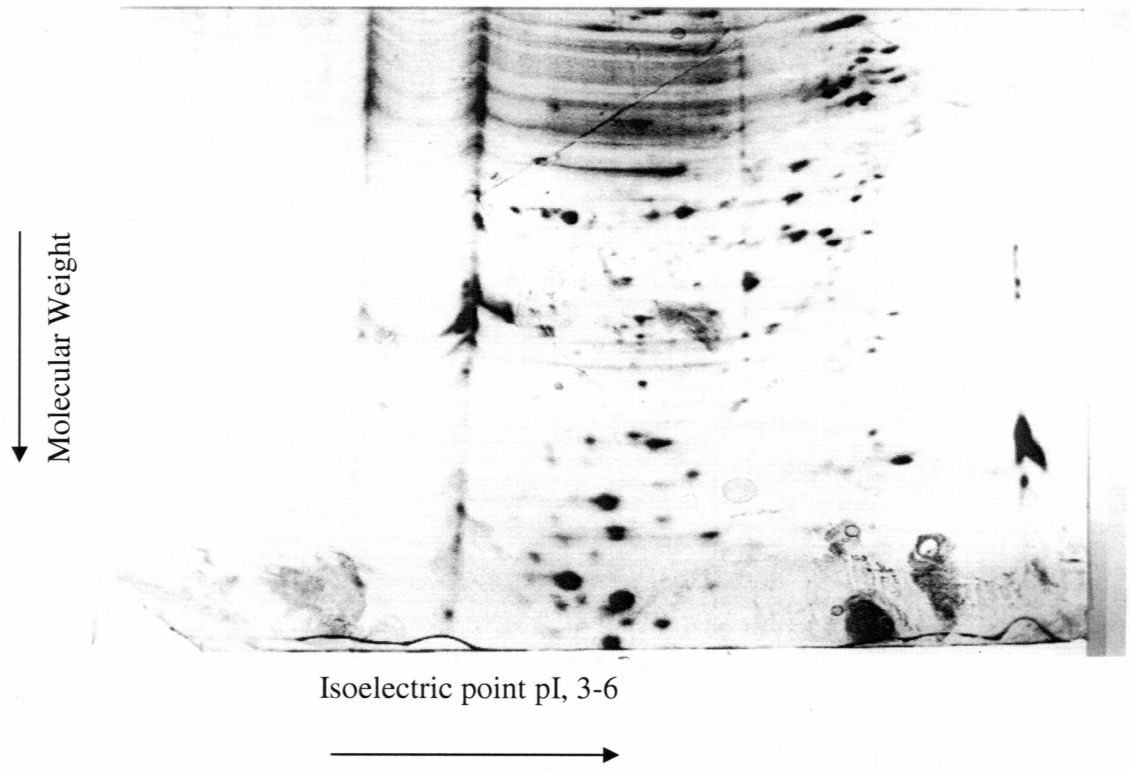

Fig 2. Cytoplasmic proteins from putative RB2256 grown in glucose (pI 3 to 6).

The figure above shows cytoplasmic proteins from RB2256 grown in glucose separated by 2D gel electrophoresis with the X axis representing isoelectric point and the Y axis representing molecular weight.

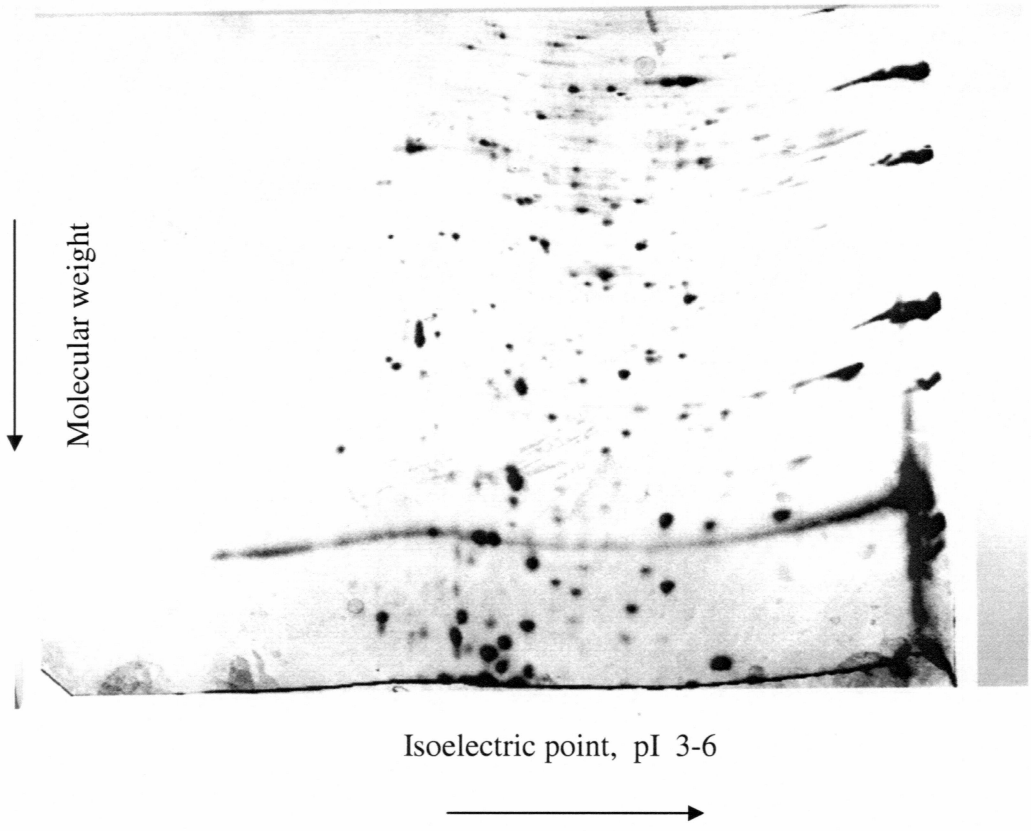

Fig 3. Cytoplasmic proteins from putative RB2256 grown in tyrosine (pI 3 to 6).

The figure above shows cytoplasmic proteins from RB2256 grown in tyrosine separated by 2D gel electrophoresis with the X axis representing isoelectric point and the Y axis representing molecular weight.

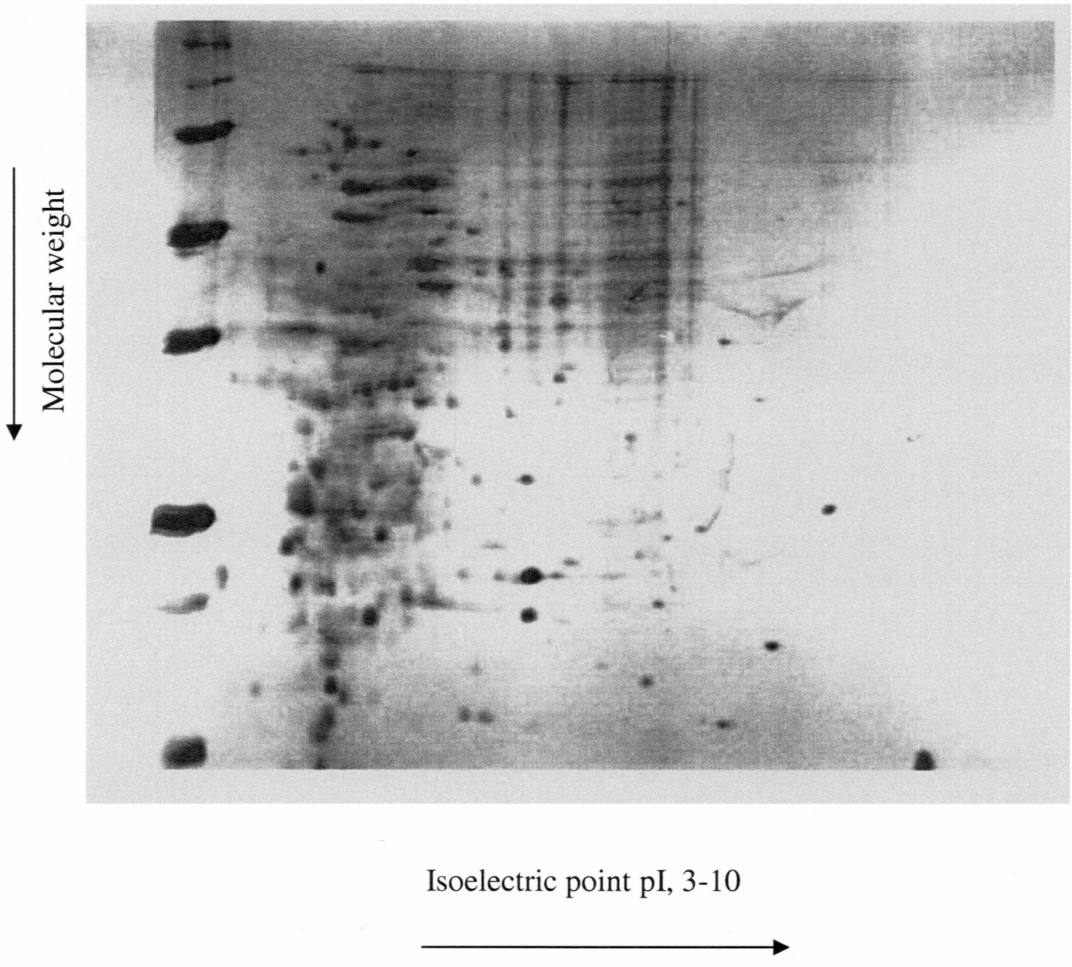

Fig 4. Membrane proteins from putative RB2256 grown in glucose (pI 3-10).

Fig 4 shows membrane proteins from RB2256 grown in glucose separated by 2D gel electrophoresis with the X axis representing isoelectric points and the Y axis representing Molecular weights. Note the numerous protein spots in Fig 4 indicating presence of large number of membrane proteins in the organism.

3.1.4 Flow cytometry

Table 2: Calculation of DNA content in putative RB2256 cultures by flow cytometry.

* denotes diploid DNA content in fg/cell

Sample	Date	Cell Mass fg/cell	DNA/cell	fg DNA/cell (Corrected for G+C= 65mol %)	Published fg DNA/cell for *Sphingopyxis alaskensis*
RB2256 Maintenance culture	27-Jan-06	70	2.8	6.2*	3.96
		40	2.0	4.6	
		80	3.8	8.6*	
RB2256 Maintenance culture	27-Jan-06	60	2.7	6.0*	
		40	2.2	4.9	
		80	3.7	8.3*	
RB2256 Glucose culture	28-Mar-06	160	1.45	3.30*	
		250	2.67	6.00*	
		80	1.2	2.7*	
RB2256 Tyrosine culture	28-Mar-06	160	1.83	4.10*	
		80	0.86	3.5*	
		150	1.62	3.70*	

The above table compares corrected DNA content (in fg per cell) of the Maintenance cultures with that of a published value of 3.96 fg/cell to ascertain purity of

the culture (Button & Robertson 2000). Note that the DNA content is low from the glucose and tyrosine cultures and that the cell mass is larger as compared to smaller cell mass and larger DNA content from the maintenance culture, however the reason is unknown . Also note that the DNA mass for *Sphingopyxis alaskensis* (RB2256) grown in maintenance culture is approximately 10 % of dry cell mass as compared to a published value of 11.1% of dry cell mass (Button & Robertson, 2000).

3.1.5 DNA Sequencing

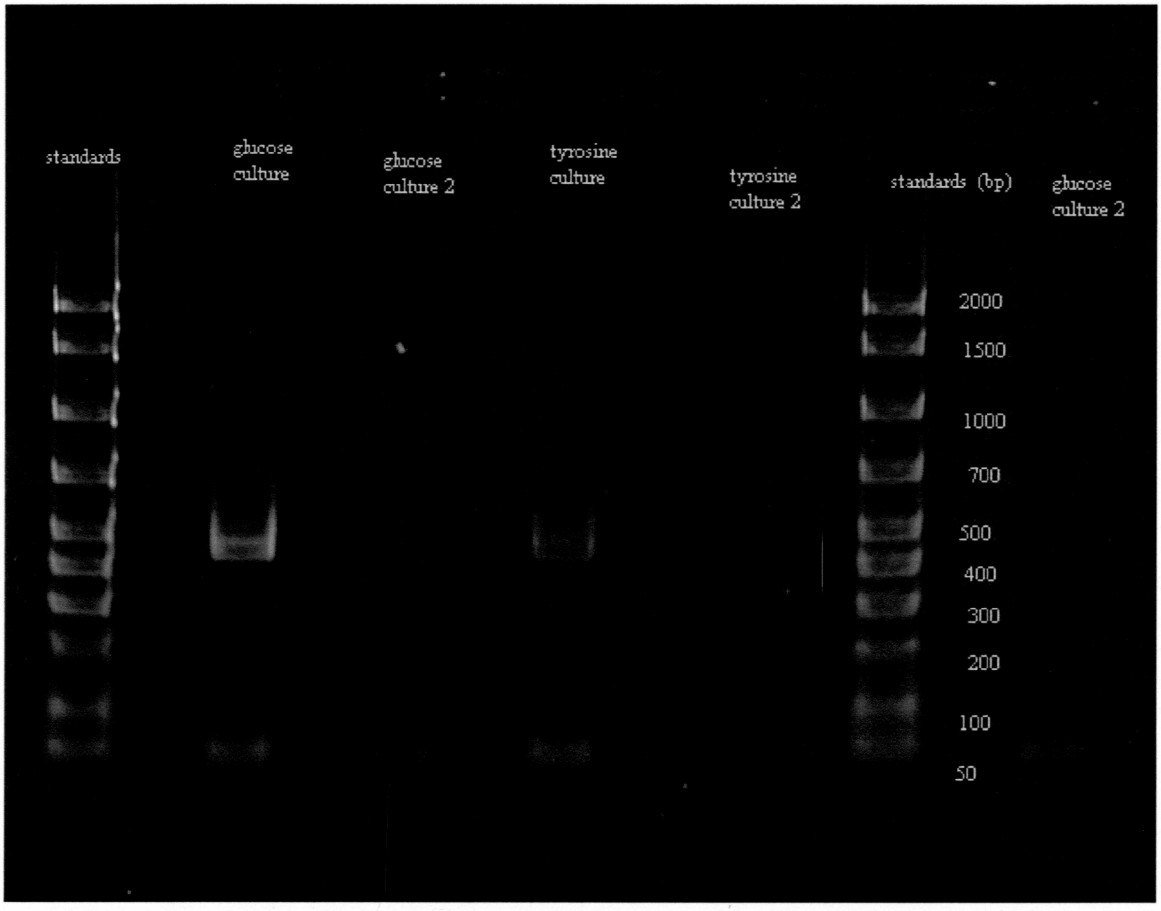

Fig 5. Agarose gel run of amplified PCR product.

Note the 441-bp band in glucose culture and tyrosine culture lanes (Fig5). The lanes, glucose culture 2 and tyrosine culture 2 do not have any relevant bands as the DNA extraction kit mentioned in methods was not used.

A 441-bp sequence obtained in the agarose gel run confirmed a successful PCR. Sequencing was done using an ABI 3100 Genetic Analyzer with a 50 cm array with polymer 6 (Pop 6). Sequencher v4.6 software was used to edit noise in the sequences. A BLAST search gave the following hit- *Brevibacterium* sp. H15 gene for 16S ribosomal RNA, isolate: H15

GAGTTTGATCCTGGCTCAGGACGAACGCTGGCTGCGTGCTTAACACATGCAAGTCGAACGCTG
AAGCCTGGTGCTTGCACCGGGTGGATGAGTGGCGAACGGGTGAGTAACACGTGAGTAACCTG
CCCCTGACTTCGGGATAAGCCCGGGAAACTGGGTCTAATACCGGATACGACYGYCGGACGCA
TGTCTGGTGGTGGAAAGTTTTTTCGGTTGGGGATGGGCTCGCGGCCTATCAGTTTGTTGGTGA
GGTAATGGCTCACCAAGACGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGG
ACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAA
ACCCTGATGCAGCGACGCAGCGTGCGGGATGACGGCCTTCGGGTTGTAAACCGCTTTCAGCAG
GGAAGAAGCGMAAGTGACGGTACCTGCAGAAGAAGTACCGGCTAACTACGTGCCAGCAGCC
GCGGTAATACGTAGGGTACGAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGTGG
TTGGTCACGTCTGCTGTGGAAACGCAACGCTTAACGTTGCGCGTGCAGTGGGTACGGGCTGAC
TAGAGTGCAGTAGGGGAGTCTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAG
GAACACCGGTGGCGAAGGCGGGACTCTGGGCTGTAACTGACACTGAGGAGCGAAAGCATGGG
GAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGTTGGGCACTAGGTGTGGGG
GGCATTCCACGTTCTCCGCGCCGTAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGTCG
CAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGCGGATTAAT
TCGATGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCAGACCGTCCTGGAAACAGGW
YYTCTTCTTTGAAGCTGGTGTACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTT
GGGTTAAGTCCCGCAACGAGCGCAACCCTCGTTCTATGTTGCCAGCACGTGATGGTGGGAACT
CATAGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCT
TTATGTCTTGGGCTTCACGCATGCTACAATGGCTGGTACAGAGAGAGGCGAACCCGTGAGGGC
AAGCGAATCCCTTAAAGCCAGTCTCAGTTCGGATCGTAGTCTGCAATTCGACTACGTGAAGTC
GGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTGAATACGTTCCCGGGCCTTGTACACA
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GGCCGTCTAATGTGGGACTGGTGATTGGGACTAAT

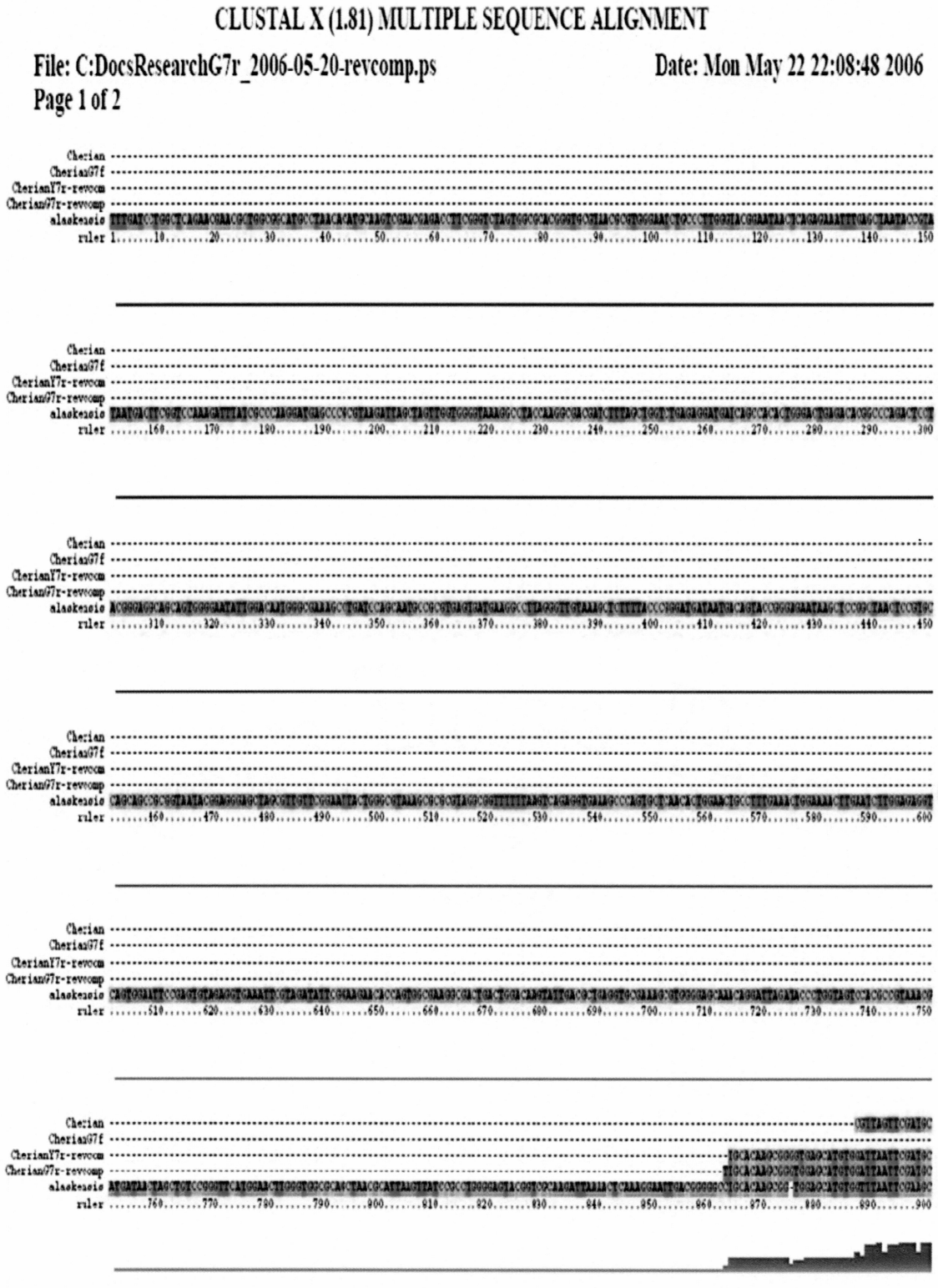

Fig 6.1 Multiple sequence alignment of 441-bp 16S rRNA gene of the putative culture with the actual organism *Sphingopyxis alaskensis* (RB2256).

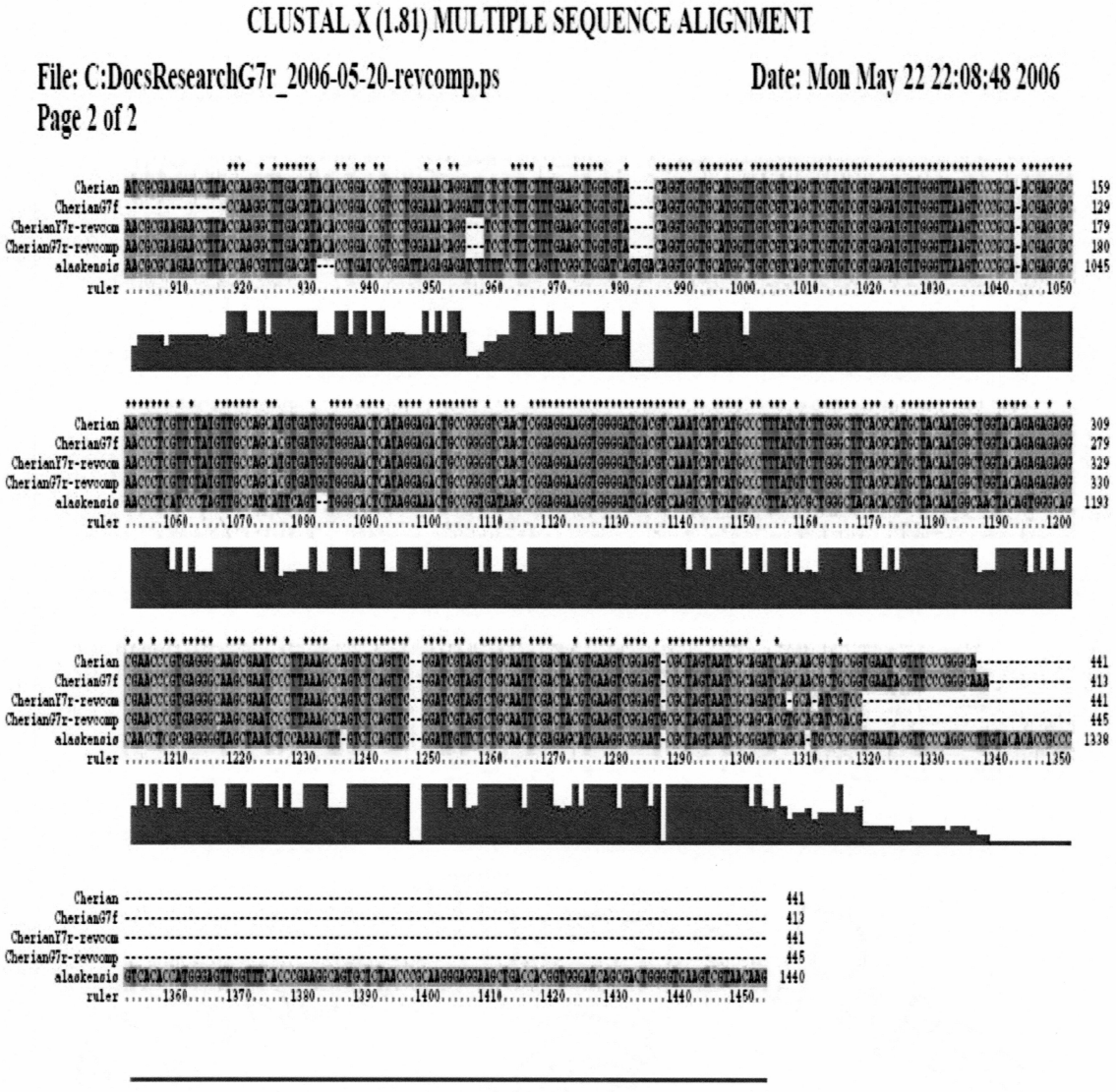

Fig 6.1 continued

Upon alignment of the above 441-bp sequence (Fig 6.1 and 6.2) with that of the real *Sphingopyxis alaskensis* 16S r RNA gene sequence using Clustal IX Multiple Alignment program it was found to be 80 % identical. About 90 sequence differences were found and a 4 bp deletion was found in the case of the *Brevibacterium* sp. relative to *Sphingopyxis alaskensis* which confirmed the presence of a significant dominant contaminant.

3.2 Discussion

Growth results from Table 1 indicate that tyrosine is the best suitable candidate as an amino acid source for growth of *Sphingopyxis alaskensis* (RB2256). Growth was evident from the turbidity of cultures. Slight turbidity was noted in the alanine, glutamine and proline cultures as well.

Presence of a pure culture was first examined by use of agar plates where uniform sized colonies were observed from both tyrosine and glucose liquid cultures. However the presence of smaller sized cell like bodies seen using epiflourescence microscopy raised the question of purity of the cultures. Because of their small size the possibility of a contaminant was dismissed and the proteomic work was continued. The electrophoretograms in Fig 2 and 3 showed unusual differential expression of protein spots for cytoplasmic fractions. Though similar spot patterns are seen while comparing the two figures, especially towards the base and center, more spots are seen in the latter. It is unclear as to how many proteins could be up- or down-regulated however it is very possible that the cultures were purely *Brevibacterium* sp. (as evident from a pure 16S rDNA sequence) or mixed cultures. Also reproducibility of protein spots between cultures was another major issue which again raised doubts of a pure culture.

Attempts made to sequence the 16S ribosomal DNA from whole cell DNA were unsuccessful and led to flow cytometry as the next alternative. Data from Table 2 seems to contradict published values for DNA content of *Sphingopyxis alaskensis* (RB2256) grown in glucose and tyrosine. The experimental values ranged from 1.35 to 3.0 fg DNA/cell, whereas the published value for *S. alaskensis* is 3.96 fg DNA/cell (Button and

Robertson, 2000). However the experimental values for the DNA content of RB2256 grown in maintenance culture range from 3.0 to 4.9 fg. Though the average was close to the ideal, the wide range of values still raised the question of possibilities of the existence of a contaminant in the maintenance culture.

Further efforts made on the 16S ribosomal gene sequencing by use of a DNA extraction kit ensured enough DNA that could be amplified as evident from the 441-bp bands in the agarose gel runs (Fig. 5). Sequencing of these bands finally confirmed a pure culture of *Brevibacterium* sp.

A reasonable explanation for the existence of *Brevibacterium* alone would be that the contaminant might have coexisted initially though in small numbers. Perhaps the substrate concentration was high due to added materials from dead cells and instigated a substrate uptake competition between RB2256 and *Brevibacterium*. Other possible reasons for such oligotrophs to succumb during challenges by high nutrition can be due to (a) Osmotic challenges as transport raises the internal osmotic pressure (b) Growth imbalance by energy depletion of ATP (c) cell insertion of inappropriate membrane proteins, (d) shut down cell phenomenon where the metabolism is irreversibly turned down by cell regulatory systems. RB2256, like other oligobacteria, have limited metabolic flexibility as compared to *Brevibacterium* or copiotrophs like *Escherichia coli.* A much lower concentration of substrate would have thus essentially diminished the contaminant population. However it is to be noted that there are reports of survival of *Brevibacterium* under carbohydrate starvation conditions and drying for extended periods

(Boyaval et al., 1985) though they are capable of growing in a complex medium like yeast extract (Wang et al., 2002).

Brevibacterium sp. is thus a formidable contaminant and can go undetected as it has a high salt tolerating capacity (8-20%), is capable of growing in a wide pH range of 5.5-9.5 with an optimum pH of 7.0 and has a genome size of 3.2 Mbp (Lima and Correia, 2000) which is very similar to features of RB2256. Interestingly *Brevibacterium* sp. is noted for its ability to metabolize aromatic amino acids like tyrosine (Leuschner et al., 1998; Ummadi and Weimer, 2001) which is perhaps the reason for growth seen in tyrosine. Also unique about this species is that it can metabolize polycyclic and heterocyclic compounds which are usually found among fungi and are absent in the bacterial world. Comparative studies on single amino acids as substrate indicate potential permeases that could be present in the inner membrane. Experimental results suggest tyrosine, alanine and glutamic acid permeases could be present as there was more growth/turbidity in these cultures unlike the other amino acid cultures

Typical oligobacteria are notoriously difficult to isolate. Furthermore, once isolated they are difficult to sustain. Strategies are constrained by limited knowledge of their nutrition and cytoarchitecture. Some facts are discussed below.

1. Bacterial permeases are perhaps numerous or non specific. An estimate of the transporter copies per transporter type is of the order of 100 from the dioxygenase content of *Cycloclasticus oligotrophus* (Button et al., 1998), however the number of transporter copies per cell is still unknown for any organism.

2. Likely substrates are numerous which would include the hundred or so macromolecular monomers and metabolic intermediates of organisms which together form the dissolved organic matter. This mixture can be the UV-photolyzed remains of structural material from autotrophic microorganisms or even non-polar substrates such as terpenes and hydrocarbons from coastal organisms.

3. Molecular complexity of the substrate in dissolved organic matter is likely great as inferred from slow growth and reduced yield (Button, 2004).

4. Cytoplasmic enzyme concentrations are possibly low. This is probably because of the small cell size as compared to the content of DNA thereby increasing the membrane protein/ cytoplasmic protein ratio for efficient substrate uptake.

5. Many species can exploit growth conditions specific to oligobacteria and are therefore vulnerable to contamination and difficult to grow as pure cultures. This is shown from the properties of *Brevibacterium* such as high salinity tolerance and survival under carbohydrate stress.

6. Most oligobacteria can use simple substrates; however this can encourage growth of contaminants.

7. Some oligobacteria such as *Cycloclasticus oligotrophus* can sustain on a single substrate like toluene as evident from the large specific affinity value (Button et al., 1998). This value when compared with specific affinity values of copiotrophs such as *Escherichia coli* grown in single substrate is noteworthy.

8. Due to the constant chemical composition of the aquatic environment oligobacteria tend to rely more on kinetic control rather than metabolic control.

9. Unknown RNA content is probably low as few ribosomes are required for slow growing organisms.

10. Quorum sensors are possibly helpful as evidenced by the aid of lactones in culturability of marine bacterial culture (Bruns et al, 2002).

These results also stress the essence of culture purity in comparative proteomics studies in microbial systems, without which the probability of artifactual data persists. Agar plate cultures are heavily relied upon currently; however, as is evident here many marine bacteria do not grow on solid media. It is thus essential to use multiple techniques such as microscopy, flow cytometry and genomics to ensure culture purity. Peptide specific species identification (Lopez et al., 2002) is a recent method enabled by the advent of mass spectrometry that could be used for rapid confirmation of culture purity.

Studies on the phylogeny of bacteria in ecosystems using hybridization with probes against portions of the 16S rDNA have shown that there are myriad organisms in nature that we cannot grow in the laboratory (Pace, 1997). However over the past decade, novel isolation strategies have brought the uncultivable numbers down to one tenth. In conclusion, a much better understanding of bacterial cytoarchitecture using proteomics tools and substrate transport kinetics is crucial to determine strategies necessary to culture the vast majority of the microbial world. Experiences here pave the way for further

physiological examination of these numerically dominant carbon recyclers, which is still now lacking due to cultivation difficulties.

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Appendix A

Artificial Sea Water (ASW) Basal Medium

2 liters of ASW Basal Medium was made up with the following recipe:

2 liters distilled water

NaCl - 60.1g

$MgCl_2.6H_2O$ - 2.06g

Na_2SO_4 - 8.02g

KCl - 1.4g

$CaCl_2$ - 0.3g

NH_4Cl - 1.02g

$NaHCO_3$ - 0.402g

KBr - 0.205g

$SrCl_2$ - 0.081g

H_3BO_3 - 0.056g

MOP - 4.22g

Adjusted pH to 7.68 and autoclaved. Labeled and stored in refrigerator

Autoclaved sealed 2mL ampules at 121^0C for 10 minutes

Appendix B

Preparation of Vitamin Premix

Prepared 1Liter primary stock of the following

Vitamin B_6 - 1mg/L

Vitamin B_{12} - 1mg/L

Biotin - 1mg/L

Secondary stock was made from the 3 primary stocks above

in 250 mL of distilled water

Vitamin B_6 - 0.25 mL

Vitamin B_{12} - 0.025 mL

Biotin - 0.0025 mL

Autoclaved the secondary stock at 121^0C for 10 minutes and then bottled in small vials and labeled as Vitamin premix.

Final concentrations of the vitamins would be

Vitamin B_6 - 1µg/L

Biotin - 0.01µg/L

Appendix C

Preparation of 1M KH_2PO_4

KH_2PO_4 - 6.805 g

Distilled Water- 50 mL

Autoclaved and sealed 2mL ampules at 121^0C for 10 minutes.

Appendix D

Preparation of Trace Mineral Mix (in milligrams per liter of distilled water)

Na_2EDTA	1000
$FeCl_3 . 6H_2O$	2000
$LiCl$	1000
$AlCl_3$	50
$NaVO_3 . 14H_2O$	5
$K_2Cr_2O_7$	0.15
$MnCl_2.4H_2O$	80
$CoCl_2 . 6H_2O$	5
$NiCl_2 . 6H_2O$	20
$CuCl_2$	20
$ZnCl_2$	60
$Na_2SeO_3 . 5H_2O$	15
$RbCl$	150
$Na_2MoO_4 . 2H_2O$	75
$SnCl_2 . 2H_2O$	1.5
KI	80
$BaCl_2 . 2H_2O$	50
$Na_2WO_4 . 2H_2O$	15

Appendix E

Oligonucleotide Data sheet (primers for 16SrDNA sequencing)

Seq Name	Sequence 5`-3`	Purif.
EUBf933	GCACAAGCGGTGGAGCATGTGG	HPLC
EUBr1387	GCCCGGGAACGTATTCACCG	HPLC

Appendix F

Source of chemicals & supplies

Bacteriological agar, casaminoacids, nutrient broth were from DIFCO, KS. PCR nucleotide mix, Thermophilic DNA polymerase 10x reaction buffer, Taq DNA polymerase, TE buffer were from Promega, WI. DNA easy extraction kit was from Qiagen,CA. BigDye and sequencing buffer were from the DNA Core Lab University of Alaska Fairbanks. IPG strips, mineral oil, ammonium persulfate, TEMED, glycine, silver nitrate and isobutanol-water mix were from BioRad, CA. Acrylamide, bis acrylamide, DNase and RNase, Tris base, sodium thiosulfate, sodium acetate, silver nitrate, sodium carbonate, Triton X-100, glycine, alanine, valine , phenylalanine, methionine, leucine, ileucine, proline, glutamine, lysine, tyrosine, arginine, serine, histidine, tryptophan, cysteine, asparagine, threonine, glutamic acid, aspartic acid, HEPES buffer, glycerol, EDTA, formamide, sodium phosphate, Sephadex G-50, ethidium bromide, Formaldehyde, TBE buffer, MgCl2 , KH2PO4 , Agarose, D-glucose, Urea, SDS, Tris-HCl and DTT were from Sigma, MO.